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Review

Genetics and ecology of the *Entoleuca mammata-Populus* pathosystem: Implications for aspen improvement and management

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ABSTRACT

Quaking aspen is damaged and killed by many pathogens but throughout most of its range *Entoleuca mammata*, the cause of Hypoxylon canker is the most serious. Hypoxylon canker has been the subject of study for over 85 years, yet gaps in our understanding of this disease remain and practical control measures for existing stands are lacking. Numerous interacting host and pathogen factors have complicated investigations and have resulted in conflicting results among researchers. This synthesis of the literature examines our knowledge of the genetics of the host and pathogen and the biology of the disease. Regenerating dense stands and selecting superior, disease resistant clones with pre-infection resistance mechanisms based on long-term field trials are strategies most likely to be effective in minimizing losses caused by this disease.

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Contents

1. Introduction

Entoleuca mammata (Wahlenberg) J.D. Rogers & Y.-M. Ju (syn. *Hypoxylon mammatum* (Wahlenberg) P. Karst., the causal agent of Hypoxylon canker, is one of the most damaging pathogens of quaking aspen (*Populus tremuloides* Michx.) in North America. Anderson (1964) estimated that in the Lake States, Hypoxylon canker kills 1–2% of the standing aspen volume or 31% of the net annual growth. A survey of aspen in the Lake States in 1971 revealed that 12.1% (range 0–40%) of the live trees were infected and the estimated tree mortality at that time would result in 4.4 million U.S. dollars per year at harvest (Marty, 1972). The market

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value of aspen can vary yearly and geographically within its range, however, the economic importance of aspen without question has increased since that study. During a 47-year study in a Minnesota stand of aspen over 60% of the tree mortality due to Hypoxylon canker occurred in the first 20 years and over 90% within 30 years after the regeneration clear-cut illustrating that this disease is most serious in relatively young stands (Ostry et al., 2004).

In North America, quaking aspen grows on sites that vary widely in soil type, topography, climate and associated tree species (Perala, 1990). This variation and high genetic diversity among aspen clones (Copony and Barnes, 1974) have contributed to the complexities of this disease and the various results obtained by different researchers examining the same aspect of the disease across its range. Reporting the results of a large-scale, long-term study of Hypoxylon canker Anderson (1964) wrote "Although analyses of the data have provided substantial information, the results in general are disappointingly ambiguous and suggest an unexpected complexity of interacting biotic and environmental factors affecting initiation and development of the disease." Based on this report subsequent research was focused on the infection process in individual trees and the development of the disease in forest stands.

One aspect of Hypoxylon canker that has complicated its study is that it can manifest itself as a twig and branch canker in the upper crowns and as a canker anywhere along the stem of affected trees. Progress in elucidating the mode of infection and other factors of the disease cycle may have been impeded by investigators primarily studying only advanced stem cankers in the early years of Hypoxylon canker research.

Manion and Griffin (1986) highlighted the critical gaps in our knowledge of the Hypoxylon canker disease cycle that at that time had been studied for 65 years. In the 22 years since that review investigators have made progress in filling several of those knowledge gaps. This research has revealed many complex interactions between the fungal pathogen and its host (Ostry and Anderson, 1990; Manion and Griffin, 1992). Applying this new knowledge should improve our success in selecting and breeding for canker resistance, managing plantations and regenerating native stands to minimize damage caused by this disease.

In this review of the Hypoxylon canker literature we examine and synthesize the findings of host resistance, pathogen biology and virulence, insect wounds as infection courts, and the relationship of stand density to spatial resistance. We make recommendations based on these findings for future aspen improvement and management.

2. Factors related to the pathogen

2.1. Taxonomy

The fungus causing a canker of aspen trees was first described by Klotzch (1833) as *Sphaeria pruinata*. Saccardo (1882) assigned it to *Rosellinia pruinata* and Cooke (1883) transferred the fungus to the genus *Hypoxylon*. Ellis (1883) described a fungus from poplar as *H. holwayii* but later considered it a synonym of *H. pruinatum* (Ellis and Everhart, 1892) and it was known as *H. pruinatum* (Kl.) Cke. until Miller (1961) combined several *Hypoxylon* species as *H. mammatum* (Wahl.) Mill. Miller's concept of the species was based on morphological traits and included morphologically similar saprophytic and weakly pathogenic species as well as the aspen canker fungus. The aspen canker fungus caused cankers on *P. tremuloides*, *P. grandidentata*, *P. tremula*, and some *Salix* species and occasionally on *P. balsamifera*. Other members included in Miller's combined *H. mammatum* species group caused cankers on *Acer*, *Alnus*, *Betula*, *Carpinus*, *Fagus*, *Picea*, *Pyrus*, *Sorbus*, and *Ulmus* (Miller, 1961). While not disagreeing with Miller's morphological concept of the species, the fact that the aspen canker fungus was included with several nonpathogenic species caused many forest pathologists to favor Cooke's (1883) classification.

More recently, Rogers and Ju (1996) described *H. mammatum* as *Entoleuca mammata*. The authors stated they accept Miller's species concept for *H. mammatum* but thought that the aspen canker species was not a *Hypoxylon* and should be placed in the genus *Entoleuca*. The genus *Entoleuca* was first described in 1922 by Sydow and Petrak describing *E. callimorpha* (Sydow and Petrak, 1922) but this fungus was considered a synonym of *H. mammatum* by others (Rogers and Ju, 1996). Several histological and microscopic features led Rogers and Ju (1996) to remove the aspen canker fungus, the only true pathogen, from the genus *Hypoxylon*. This was supported by Mazzaglia et al. (2001) who used 5.8S and ITS sequences of rDNA to examine the genetic relationships among *Hypoxylon* and related genera. However, they did not include *E. callimorpha*, the type species of *Entoleuca* so molecular evidence of its relationship to *H. mammatum* is lacking. Rogers and Ju (1996) noted that if fresh collections of *E. callimorpha* were shown to differ biologically, a new genus for *H. mammatum* would be required.

2.2. Host range

Several genera of hardwood trees have been reported as hosts of *E. mammata* (Miller, 1961) but conclusive evidence for confirming saprophytic or pathogenic relationships on many of these hosts is largely lacking. Quaking aspen is commonly the most severely affected species in North America with *P. grandidentata* occasionally infected. *E. mammata* isolates from alder (*Alnus* spp.) and willow (*Salix* spp.) were not pathogenic on aspen (French et al., 1969). Isolates from some species of willow have, however, been reported to be pathogenic to various degrees on quaking aspen (French and Juzwik, 1984; Bucher and French, 1989), but an isolate from *P. tremuloides* was not pathogenic on species of *Salix* (Bucher and French, 1989). The only reports of cankers on *P. balsamifera* caused by *E. mammata* were from New York by Povah (1924), in the Lake States by Lorenz and Christensen (1937) and from Saskatchewan and Alberta by Bier (1940). *Populus deltoides*, *P. balsamifera*, *P. balsamifera* × *P. deltoides*, and *P. alba* × *P. deltoides* were resistant to inoculations with mycelium of *E. mammata*; however, excised branches of *P. deltoides*, *P. balsamifera* and hybrids between these species did become infected when inoculated (Berbee and Rogers, 1964). In another field trial, inoculated *P. deltoides*, *P. balsamifera* and *P. deltoides* × *P. balsamifera* resisted infection (Rogers, 1963). Cankers caused by *E. mammata* were found on *P. nigra* var. *betulifolia* × *P. nigra* 'Volga', *P. nigra* var *betulifolia* × *P. trichocarpa*, *P. maximowiczii* × (*P.* × *berolinensis*), *P.* 'Candicans' × (*P.* × *berolinensis*), and *P. deltoids* × *P. nigra* 'Incrassata' (Ostry and McNabb, 1986). Anderson et al. (1960) reported Hypoxylon canker on *P. tremula* growing in Wisconsin. In Europe P. *tremula*, *P. alba*, *P. trichocarpa* and the hybrid *P. tremula* × *P. tremuloides* have been reported as hosts (citations in Kasanen et al., 2004). These accounts underscore the need for additional inoculation experiments that take into account the variation in pathogenicity of the fungus isolates used and genetic variability in the host genotype tested.

2.3. Disease distribution

Hypoxylon canker was first described from New York and Michigan in a paper by Povah (1924) who mentioned a report of the disease from Maine as well. Also in that paper, Povah cited a paper (Hartley and Hahn, 1920) that included a photograph of an unknown canker disease on aspen from 1909 in New York that

clearly resembled Hypoxylon canker. Povah suggested that Hypoxylon canker of aspen was probably present throughout the northeastern United States. Presently, in the northeastern and Lake State regions of the United States Hypoxylon canker is common and one of the most damaging diseases of aspen (Sinclair and Lyon, 2005). Hypoxylon canker on aspen is less important in the western United States (Hinds, 1985) and absent in Alaska (Hinds and Laurent, 1978). In Canada the disease is widely distributed (Bier, 1940) and the disease on *P. tremula* in Europe was described from Finland, Germany and Sweden (Miller, 1961). The disease was first noted in France in 1975 (Pinon, 1979) and isolates of the pathogen have since been collected in Italy and Switzerland (Kasanen et al., 2004).

2.4. Role of spores in the infection process

Knowing the function of spores of the pathogen is important in understanding the biology of the disease and for guiding a strategy for aspen improvement. Two spore types are formed. Ascospores are the result of meiosis. The other spores have been called conidia and although their function was thought to be asexual spores in infection, they are now thought to function as spermatia for the production of ascospores.

The conidial stage of *E. mammata* was first described by Ponomareff (1938) who reported obtaining cultures from germinating conidia. Conidia are borne on specialized coremia commonly known as hyphal pegs that form within diseased host tissue and push up the periderm exposing the spores (Bier, 1940). The role of these spores in the disease cycle has long been uncertain due to the conflicting results among investigators regarding their ability to germinate and function as asexual spores. Gruenhagen (1945) reported culturing conidia and obtaining cankers on trees inoculated with the cultures. However others (Anderson and French, 1972a; Bier, 1940; French and Manion, 1975) reported that only occasionally would trees inoculated with cultures from germinating conidia result in cankers.

In experiments that separated conidia from hyphal fragments using centrifugation and filtration techniques, the spores germinated but did not develop further (Rogers and Berbee, 1964) or only a few colonies developed (Bagga et al., 1974; Griffin et al., 1992). Inoculation of plants with conidial suspensions minus any hyphal fragments failed to produce cankers (Bagga and Smalley, 1974b). Results of these studies strongly support the hypothesis that these spores are not infective and do not propagate the fungus in the field.

Several insects that live in and feed on Hypoxylon cankers have been described and may be involved in inadvertently moving conidia from one canker to another, resulting in spermatization. Graham and others (1963) mentioned that the Hypoxylon canker beetle *Astyloposis macula* (Say) (=*Amniscus macula* (Say) was commonly found feeding on diseased bark. The closely related beetle *Astyleiopus variegates* (Haldeman) was commonly found feeding on mycelium and spores of *E. mammata* (Ostry and Anderson unpublished). Kukor (1979) reported that the beetle *Enicumus aterrimus* Motschulsky was frequently found in Hypoxylon cankers where larvae and adults were seen to feed on mycelium and spores of *E. mammata*. Viable spores and mycelium were recovered from the beetles and adults were observed to move from tree to tree.

Ascospore discharge from a single perithecium was observed in a darkened room by Bier (1940) who found that up to 61 asci discharged some 488 ascospores per minute. Froyd and French (1967) noted that ascospore discharge occurs on a 12-h cycle with peak collections recorded from 10 p.m. to 10 a.m. They also found that ascospores continued to be liberated from cankers on trees felled and placed on the forest floor for up to 23 months. Wood and French (1965a) reported that cankers on the south side of trees enlarged and ascospores were ejected from perithecia during winter months in Minnesota. It was suggested that ascospore infection of aspen may occur during the dormant season when host bark turgor was low (Bier and Rowat, 1962b); however, this has not been demonstrated.

Bier (1940) isolated the eight single ascospores from each of 2 asci and established 16 single ascospore cultures on nutrient agar. The eight cultures from each ascus consisted of four morphologically distinct groups. Pairings of the two isolates of each group resulted in merging of the two colonies into a single colony. Pairings between isolates from different groups resulted in a zone of aversion or demarcation between the two colonies. This was the first indication that a somatic incompatibility system and an outbreeding mechanism was operating in this species. The results of Griffin et al. (1992) intra ascus pairings of single ascospores were the same as Bier's (1940). All inter-ascus pairings of single ascospore cultures resulted in a demarcation zone and none of isolates in these pairings merged.

To determine if nuclear exchange can occur between field isolates of *E. mammata*, Sharland and Rayner (1989) paired six field isolates in all 15 possible combinations on nutrient agar. From the aversion zone of each pairing they established 10 colonies from conidia and hyphal fragments. The 10 colonies from each pairing were then paired with both parent isolates. If nuclear exchange had occurred, the 10 isolates would have contained nuclei from both parents, and an aversion zone would have occurred when paired with both parents. However, in all of the pairings of the 10 isolates individually from each of the 15 parent isolate pairings, an aversion zone occurred only with one or the other parent, never both, indicating all 150 isolates from the aversion zones were haploid. This somatic incompatibility system, first noted by Bier (1940) explains why cankers in nature are the result of infection by a single ascospore, and also why pathogenic races of this pathogen do not and cannot occur in nature.

Ascospore germination was inhibited both on unwounded and wounded (only phellum layer removed) aspen bark but inhibition was greater on freshly wounded bark (Hubbes and D'Astous, 1967). This work supported the findings of French and Oshima (1959) and was further evidence of the importance of wounds completely through the bark for infection to occur (discussed in Section 3.3).

Gruenhagen (1945) produced cankers using ascospores placed into wounds made into the xylem of aspen stems. Before inoculation the bark and cambium tissues around the wounds were killed by pounding the area with a blunt tool. Shea (1963) readily obtained cankers using mycelium to wound-inoculate trees but failed to induce cankers using mixtures of ascospores and conidia. Anderson and French (1972b) inoculated young aspen through various age drill wounds with ascospore suspensions and demonstrated that a mean of 1% (range 0–7) of the ascospores germinated in the sapwood but the experiment was not designed to study whether cankers would develop at these wounds. In another experiment, *Entoleuca mammata* was recovered from the tops of untreated, but not surface disinfected aspen stem sections that had their ends coated with paraffin and placed in moist sand in a greenhouse (Anderson and French, 1972a). However, surface disinfected stem sections inoculated with ascospores resulted in canker formation at the top of the sections under the paraffin but not on stem sections that were not dipped in paraffin. The authors suggested that the fungus may be present as spores or mycelia on or in the sections but they did not offer an explanation for the lack of development on sections not dipped in paraffin (Anderson and French, 1972a).

Infection with ascospores has been reported with inoculations of plants in a greenhouse if spores were suspended in fungal extracts (Bagga and Smalley, 1974b) and when moisture-stressed tissue culture plantlets were inoculated with ascospores (Bélanger et al., 1989b). Ostry and Anderson (1995) demonstrated infection of trees with ascospores in the field by inoculating branch galls on *P. tremuloides* caused by *Saperda inornata*.

2.5. Fungal population structure

Population studies of *E. mammata* from cankers on aspen in a plantation were carried out by Griffin et al. (1992). They obtained mycelial isolates from the margins of individual cankers. When isolates from a single canker were paired on nutrient agar, all of the isolates merged. When isolates from different cankers were paired, aversion zones occurred at the interface in all pairings. These results indicate that cankers result from a single ascospore and that somatic incompatibility keeps these isolates genetically isolated and unique. Ostry and Anderson (unpublished) recovered *E. mammata* in pure culture from 113 oviposition wounds made by treehoppers (*Telemona tremulata*) on branches of 12-year-old aspen in a Minnesota plantation in 1996. Cultures were recovered from 1 to 17 oviposition wounds on each of 67 trees. Intra-tree pairings between 31 isolates paired in 42 different combinations and inter-tree pairings between 57 isolates paired in 160 different combinations yielded no merged colonies, indicating all wounds were colonized by genetically unique isolates resulting from ascospore infection, similar to the findings of Griffin et al. (1992).

Kasanen et al. (2004) made a sequence analysis of 2 DNA markers from 27 isolates of the fungus from North America and found that each isolate was a unique genotype and the authors concluded that the fungus is actively outcrossing and that asexual propagation does not occur. Ascospores are the main source of inoculum of the fungus but infection requires wounds through the bark (discussed in Sections 3.2 and 3.3).

2.6. Host colonization

Bagga and Smalley (1974a) were first to report wide variability among *E. mammata* isolates in culture morphology, conidia production, growth rate *in vitro*, and virulence. Study of a population of *E. mammata* isolates collected from eight locations revealed that glucose was the best carbon source for fungal growth and that the inability to metabolize sucrose (Bagga, 1968) was not related to isolate virulence (Anderson and Schipper, 1975). Isolate growth *in vitro* varied but growth rates did not predict virulence in inoculated trees of a single clone. Based on the large variation within isolates collected from the same area and between isolates from the various collection sites the authors (Anderson and Schipper, 1975) concluded that many biotypes of *E. mammata* existed. This report also provided further evidence that fungus growth occurs first in the wood where it utilizes glucose and only later in the bark. French and Manion (1975) also demonstrated variation in the ability of cultures to produce cankers based on a set of five aspen clones that varied in susceptibility to the isolates but no single isolate produced the largest canker on all clones. Isolates collected from *P. tremula* in France exhibited variation in culture morphology and growth over a range of temperatures similar to those reported in the United States (Pinon, 1979).

The period of colonization of aspen tissue by *E. mammata* before symptoms develop has been called both a latent and endophytic phase. We chose to call the period from inoculation of *S. inornata* galls with ascospores until symptoms develop (average 24 months) a latent period (Ostry and Anderson, 1998). When cross-sections were made of these infected, asymptomatic 1- and 2-year-old galls, areas of decayed xylem and islands of necrotic phloem tissue were evident. Scanning electron microscope studies of these galls revealed sheets of mycelium connecting the diseased xylem and phloem tissue (Ostry and Anderson, 1998). The pathogen cannot utilize sucrose; therefore the energy needed to detoxify the phenolic compounds in the bark must come from decay of cellulose in the xylem. The mycelial sheets transfer the energy from the xylem to the pathogen in the bark tissues.

Chapela (1989) was the first to suggest that *E. mammata* commonly occurs as an endophyte of unwounded healthy aspen and that fungal growth and symptomatic canker development depends on decreasing water content of the wood. The fungus was isolated from a low percentage of wood samples of 45% of the 11 healthy trees tested. *E. mammata* was recovered from wood samples with bark but not from wood samples that had the bark removed, indicating latent colonization in the bark or near the cambial region. As previously discussed, in an earlier study Anderson and French (1972a) reported that Hypoxylon cankers developed under the paraffin-coated tops of aspen stem sections collected from healthy trees, set in moist sand and incubated in growth chambers. The authors concluded that *E. mammata* spores or mycelia were present on or in the living, healthy trees.

Common to these two studies is the inference that the fungus was already growing in the stems as an endophyte, or was on the stems as spores or mycelium and that further development by the fungus was determined by decreasing wood moisture, similar to species in the genus of *Biscogniauxia* (Nugent et al., 2005). Further work along this line was done by Manion and Yuan (1992) using wounded and non-wounded moisture-stressed potted aspen plants that were inoculated with ascospores. They isolated the fungus from asymptomatic green tissues and suggested this demonstrated an endophytic phase of the pathogen. A reduced moisture content of host tissue may not always be necessary for fungal growth as Hutchison (1999) reported isolating *E. mammata* from the sapwood of an apparently healthy aspen tree that had not undergone a drying treatment. In these studies the term endophyte was used to describe growth of the fungus where no host wounds were described. The term endophyte, as defined by Wilson (1995), can be used to describe the latent phase that precedes symptom development, however, further study is needed to determine how spores or mycelium of *E. mammata* on unwounded aspen mediated by low bark moisture results in infection and canker development.

Griffin et al. (1986) demonstrated that hyphal growth rates *in vitro* are stimulated by the amino acid proline and that drought-stressed aspen produce proline in concentrations which the authors hypothesized may be related to the enhancement of canker elongation in trees. In inoculation tests, any factor increasing host moisture stress increased susceptibility (Bagga and Smalley, 1974c). Susceptibility of water-stressed tissue culture plantlets inoculated with ascospores was also increased (Bélanger et al., 1989b). Reductions in the levels of compounds inhibitory to *E. mammata* were thought to be responsible for the greater susceptibility of water-stressed tissue cultured plantlets (Kruger and Manion, 1994). Amino acid content of water-stressed tissue culture plantlets was found to be related to the canker susceptibility of five aspen source clones (Bélanger et al., 1989c). The authors also suggested that elevated levels of nitrogen compounds of some water-stressed aspen clones increased their susceptibility to *E. mammata*.

2.7. Toxin production

Experimental evidence that *E. mammata* produced substances that were toxic to living aspen tissues was provided by Hubbes (1964). These diffusible substances inhibited the normal toxic

effect of bark to the growth of the fungus and prevented callus production at the wound site. Partially purified and crude toxic metabolites produced by *E. mammata* used in various assays in attempts to demonstrate their role in pathogenesis and in examining differences in host resistance have yielded mixed results (Bruck and Manion, 1980a; Griffin et al., 1980; Pinon, 1986; Bodo et al., 1987; Stermer et al., 1984; Griffin and Manion, 1985; Bélanger et al., 1989a; Mottet et al., 1991; Pinon and Manion, 1991; Kruger and Manion, 1993a,b).

Bagga and Smalley (1974b) reported that inoculations of aspen plants in the greenhouse with ascospores resulted in canker development only if cell-free extracts from mycelial cultures were applied to the wounds at the time of inoculation, presumably preventing the callusing of the wound. Schipper (1978) reported that extracts of ascospores did not contain toxic substances. *E. mammata* isolates differed in the amount of toxin produced with a nonpathogenic conidial isolate producing a barely detectable amount (Schipper, 1978). Wann (1985) used culture filtrates to screen aspen plant cultures *in vitro* and plants regenerated from surviving cultures, established in soil and challenged with mammatoxin retained the resistance expressed *in vitro*. Additional bioassays of tissue culture plantlets of different genotypes with culture filtrates suggested that resistance in this pathosystem is controlled by several genes and that a toxin bioassay alone is not adequate to screen aspen for canker resistance (Kruger and Manion, 1993a). At this time the role of toxic *E. mammata* metabolites remains unclear owing to the many complex genetic interactions in this disease system (Kruger and Manion, 1993b).

3. Factors related to the host

3.1. Clonal variation in resistance

Variation in the incidence of Hypoxylon canker was observed among clones of *P. tremuloides* (Copony and Barnes, 1974). This was the first study to examine the distribution of the disease and disease severity among clones as sampling units. As Manion and Griffin (1992) pointed out, the difficulty in using single observations of the amount of canker in natural clones of aspen is that individual ramets can die and decompose or survive with the disease and thus the number of diseased individuals (living and dead) changes over time. Forty aspen clones were monitored for canker incidence and tree mortality in a 16 ha stand in northern Minnesota for 47 years (Ostry et al., 2004). Canker prevalence was highly variable among clones at each observation period resulting in changes in clone ranking based on periodic canker prevalence. Canker incidence among clones at the end of the study ranged from 4.4 to 63.5%. Selecting clones that may be resistant to the disease based on disease prevalence (ratio of living infected trees to total living trees) at any given time may not be indicative of the long-term disease incidence based on all ramets in a clone over repeated observation periods and this likely has complicated aspen improvement efforts.

3.2. Host defense

Aspen has numerous chemical defense mechanisms active against damaging agents such as insects and fungal pathogens (Lindroth, 2001). In the earliest report of potential host defense compounds French and Oshima (1959) found that adding the green layer of aspen bark to agar inhibited the germination of *E. mammata* ascospores. The authors suggested that the type of wound and portion of bark exposed may determine if spores germinate and develop into a colony and they hypothesized that infection may occur above branch axils where the green layer of bark may be absent. Manion (1975) reported that 20 of 39 small cankers originated near the base of 1- and 2-year-old dead branches.

Pyrocatechol isolated from aspen bark totally inhibited the growth of *E. mammata in vitro* (Hubbes, 1962). Hubbes (1969) later identified benzoic and salicylic acids as additional fungistatic components of aspen bark and that these phenolic acids acted synergistically to inhibit fungal growth. Bier and Rowat (1963a) found that experimental factors related to inoculum and substrate used influenced the ability of aspen bark to inhibit *E. mammata*. A phytoalexin in exudates of freshly wounded, but not non-wounded, aspen sections inoculated with mycelial plugs of *E. mammata* was found to inhibit germination of *E. mammata* ascospores but not mycelial growth (Flores and Hubbes, 1979, 1980). Inoculation with ascospores failed to elicit phytoalexin production. The authors concluded that for the production of phytoalexins to occur in aspen, the host, pathogen and wound are equally important factors.

Several authors have reported that microorganisms are present on or in the bark of aspen that are antagonistic to *E. mammata*. Bacteria associated with perithecial stromata, freshly ejected ascospores, and from healthy aspen bark were found to inhibit ascospore germination and the authors suggested that these bacteria may prevent establishment of the fungus in aspen (Wood and French, 1965b). Of the 29 species of fungi associated with healthy bark, some inhibited and others stimulated development of the fungus. The authors suggested that under some conditions germination of ascospores in entry courts would be favored over development of antagonistic microorganisms.

The inhibitory effect of microorganisms from the bark and sapwood of healthy aspen on the growth of *E. mammata* was investigated by Bier and Rowat (1962b) who used these saprophytes to demonstrate biological control of disease development on inoculated cuttings (Bier and Rowat, 1962a, 1963b). Bier (1965) concluded that susceptibility or resistance to infection is more related to microbiological factors of the living bark than to chemical inhibitors.

Based on these studies the evidence suggests that the toxicity of the green layer of aspen bark to *E. mammatum* is an important constitutive resistance mechanism that may be augmented in some cases by inhibitory microorganisms.

3.3. Insect wounds as infection sites

Bier (1940) was the first to complete Koch's postulates, describe the fungus as a wound pathogen, and describe cankers originating in galleries on branches made by *Oberea shaumii*. Gruenhagen (1945) reported finding 14 of 1018 wounds made by *Saperda calcarata* colonized with *E. mammata*. Graham and Harrison (1954) reported finding cankers associated with wounds made by *S. calcarata*, *Dicera tenebria*, *Lepidopterous* spp., *Agrilus* spp., other Cerambycidae, Agromizidae and several unidentified insect species.

Six years after defoliation by the forest tent caterpillar (*Malacosoma disstria*) the incidence of tree mortality caused by Hypoxylon canker increased with defoliation intensity of the affected stands (Churchill et al., 1964). The authors noted a corresponding increase of wood boring insects with increasing defoliation intensity.

Hubbes (1964) was the first to describe the type of wound necessary for infection. He concluded that *E. mammata* is a sapwood parasite that requires a wound directly into the wood or deep into dying or recently dead bark to avoid the toxic compounds in the bark, become established and then invade the cambium. Bagga and Smalley (1969) described the ideal wound for infection

by the fungus as one that is deep enough to expose the xylem and provide high humidity. Several species of insects making wounds meeting these requirements have since been reported.

Although the fungus was not isolated, Nord and Knight (1972b) found that 14 of the 40 Hypoxylon cankers examined on aspen branches were associated with *O. schaumii* and *S. inornata* galleries. They suggested that expansion of cankers from these twigs to stems under favorable conditions could account for the yearly fluctuating prevalence of the disease reported by Schmiege and Anderson (1960). Manion (1975) also reported cankers originating in insect galleries, reporting that 10 of 39 cankers were associated with galleries of *S. concolor* (=*S. inornata*). From a survey of aspen clones in New York it was concluded that branches were the site of infection courts and with increasing tree size cankers occurred farther away from the main stems and thus fewer lethal stem cankers developed (Falk et al., 1989). In Minnesota the natural rate of infection through *S. inornata* galls on aspen in a plantation was 1.4% (Anderson et al., 1979). Of the cankers originating in branch galls nearly 12% expanded into the main stem. Wounds through the bark of galls made by downy woodpeckers (*Picoides pubescens*) foraging in aspen were thought to enhance the likelihood of infection (Ostry et al., 1982). The authors suggested that woodpeckers observed foraging on wood boring insects in aspen stems infected with *E. mammata* and probing on galls could vector the fungus. Over 80% of 1409 *S. inornata* galls colonized by *E. mammata* had probing and extraction wounds made by downy woodpeckers (Ostry and Anderson, 1998).

After an outbreak of the periodical cicada (*Magicicada septendecim*) in a Wisconsin plantation 7% of the oviposition wounds became infected (Ostry and Anderson, 1983). Of the cankers originating on branches in *S. inornata* galls and cicada oviposition wounds 30 and 27% of them respectively, expanded into the main stem (Ostry and Anderson, 1998). Oviposition wounds made by tree hoppers have also been associated with cankers on small twigs in the upper crowns of trees (Ostry and Anderson, 1986).

Kukor (1979) commonly found larvae, pupae and adults of the beetle *Enicmus aterrimus* under the blistered bark of cankers. The fungus was isolated from fecal material, dissected gut tissue, and the bodies of larvae and adults. Evidence was obtained that adults move around within crowns of individual trees and may move between trees suggesting the potential of this insect to move the fungus into wounds made by other insects.

Based on the above observations infection of aspen by *E. mammata* commonly occurs through insect wounds into the xylem where the fungus is not inhibited by toxins (Hubbes, 1962) in the bark. This type of wound is necessary for infection because the fungus can utilize cellulose, cellobiose and glucose in the xylem but not sucrose in the phloem (Bagga, 1968; Schipper and Anderson, 1971).

3.4. Spatial resistance

The interactions of environmental and biotic factors with tree density that influence disease incidence have been described as spatial resistance (McNabb et al., 1982). The authors state that these interacting factors are modified by stand density and thus density can contribute to increased disease resistance in some pathosystems. Stand density has been reported to influence Hypoxylon canker incidence with trees in low density stands or trees along stand edges having a greater disease incidence than well-stocked stands (Schreiner, 1925; Day and Strong, 1959; Anderson and Anderson, 1968; Copony and Barnes, 1974; Bruck and Manion, 1980b; Brandt et al., 2003). Nord et al. (1972a) reported that infestation of stems by *S. inornata* which make wounds that are often colonized by *E. mammata* was greater in small stands along roadsides and along the edges of larger stands. Early results from a long-term study revealed more infection in low density stands but later in this study this relationship was less certain, however, in another study trees growing along stand edges had a greater prevalence of cankers than interior trees (Anderson, 1964). Anderson and Martin (1981) found that defoliation of aspen by the forest tent caterpillar (*Malacosoma disstria*) and low stand density increased stem canker incidence. In all of the above studies, host genotype was confounded with stand density. However, studies of post-infection resistance mechanisms have shown these to have very low heritability rates (Valentine et al., 1976) and therefore these traits contribute little to reducing canker incidence. Spatial resistance, on the other hand, seems to be a major contributor to reducing canker incidence in natural stands by reducing potential infection courts created by sun-loving insects that prefer to oviposit in low density aspen stands.

Quaking aspen is a pioneer species invading sites via seed or root suckers after fire or other disturbances. Once established there is intra-species competition while clones develop from common root systems. After fire or harvest 25–75,000 stems per ha can regenerate within 2 years that are then gradually reduced through self-thinning and mortality caused by insect pests and disease. Studies of Cerambycid beetles in young aspen stands revealed that stocking levels below 20,000 stems per ha favored beetle populations and as trees aged oviposition wounds were more abundant on upper branches than on the main stems of trees (Myers et al., 1968). Beetle oviposition sites were almost always on branches exposed to sunlight. The spatial and temporal distribution of Hypoxylon cankers in stands and on individual trees described by many researchers is closely associated with these findings (Ostry and Anderson, 1998).

The practice of thinning aspen to increase growth and the potential for subsequent damage by Hypoxylon canker has been controversial. Some investigators reported an increase in Hypoxylon canker in thinned stands (Anderson and Anderson, 1968) and others reported no increase (Pitt et al., 2001). Anderson (1964) reported mixed results on the influence of thinning on canker incidence from two experiments with one showing significant effects and other one with less certain results. It was hypothesized that persistent branches on trees in thinned and low density stands may account for greater infection and development of stem cankers (Ostry and Anderson, 1979). In a field trial of 25 full-sib families the incidence of canker was lowest on families with good self-pruning traits (Li et al., 1993b). In a 47-year thinning study canker incidence was affected more by clonal differences than the thinning treatments (Ostry et al., 2004). One recommendation called for the delay in thinning aspen stands until the lower branches have naturally pruned (Cleland et al., 2001).

Spatial resistance may be a major reason for the success of aspen in the Lake States. Because stem density is high in young aspen stands, the importance of pre-infection resistance (Fig. 1) which is related to low insect populations and few oviposition wounds has not been recognized. Instead, efforts have been concentrated on selecting for post-infection resistance mechanisms such as limiting canker elongation, callus production and various branch characteristics. Thus, selection of parents from wild stands has not resulted in effective levels of resistance to infection when trees are planted at wide spacings in plantations (Ostry and Anderson, 1998). One potential option to avoid high canker incidence in plantations is to establish plantings at a wide spacing and then cut back the trees at age 6–10 to rapidly develop a dense sucker stand to mimic a natural, fully stocked stand.

3.5. Selection and breeding

Aspen breeding for primarily increased growth and secondarily for Hypoxylon canker resistance has been underway in the Lake States since 1955 (Li et al., 1993a). Aspen hybrid vigor has been demonstrated based on gains in height growth of interspecific hybrids ranging from 29 to 34% with estimated gains in volume growth over 100% (Li et al., 1993a). However, several of these interspecific hybrids were triploids and were found to be seriously damaged by a bark disease caused by *Lahmia kunzei* (syn. *Parkerella populi* (Funk)) not found on native aspen (Ostry, 1986; Enebak et al., 1996b). Heimburger (1966) postulated that susceptibility of several hybrid aspen to the systemic bronze leaf disease now known to be caused by *Apioplagiostoma populi* (Smith et al., 2002) was a genetic barrier to hybridization between aspen species. Natural hybrids between *P. grandidentata* and *P. tremuloides* were reported to be more susceptible to disease, including Hypoxylon canker, than either parent species (Barnes and Pregitzer, 1985).

Challenging aspen with *E. mammata* through mechanical wounds and using canker expansion or callus production that retards canker expansion to identify resistant genotypes has been attempted by inoculating seedlings (Enebak et al., 1996a), branches (French and Manion, 1975; Valentine et al., 1976; French and Hart, 1978; Griffin et al., 1984) and main stems (Enebak et al., 1999).

Greenhouse inoculations of 3- and 9-month-old seedlings of two full-sib families with a 30-year history of canker incidence in the field, demonstrated that the family susceptibility could be differentiated but correlation to natural canker prevalence in the field was highly dependent on seedling age, with the more lignified 9-month-old seedlings being better predictors than the 3-month-old seedlings (Enebak et al., 1996a). Histological evidence of two aspen genotypes varying in field resistance to canker revealed that chemical and morphological barriers to the fungus varied by age of tissue and correlated with family history of canker incidence (Enebak et al., 1997).

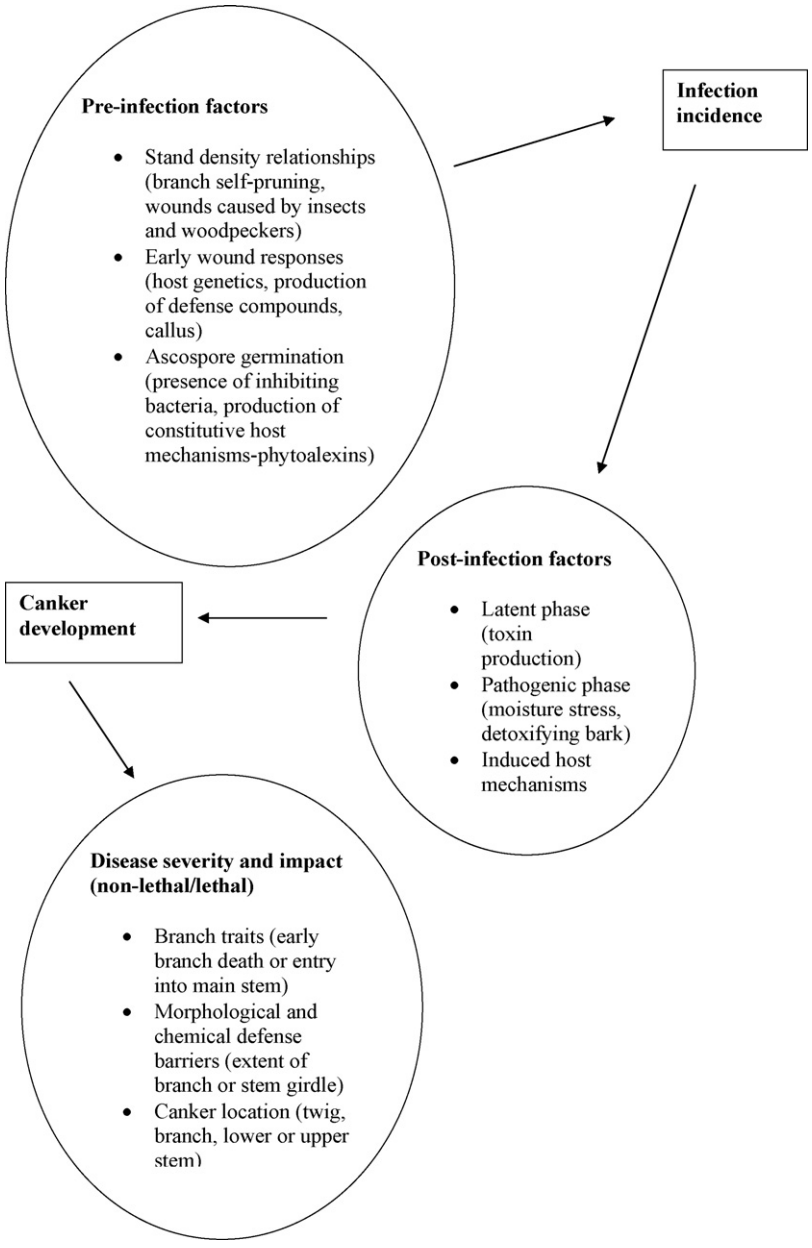

Fig. 1. Conceptual model of the interacting factors in the Hypoxylon canker disease cycle.

Early wound responses of wound-inoculated greenhouse-grown aspen resistant and susceptible to canker revealed differences in boundary zone formation due to activity of phenylalanine ammonia-lyase and cinnamyl-alcohol dehydrogenase and the synthesis of phenylproponoid monomers that affected compartmentalization of the fungus by a lignified barrier and a wound callus barrier rich in phenolic substances (Bucciarelli et al., 1998, 1999). In a study of defense-related genes, it was found that aspen genotype, wounding and time after wounding significantly affected the expression of wound-inducible genes with levels somewhat associated with degree of canker resistance (Thamarus and Furnier, 1998).

Field inoculations of branches on 100 aspen clones revealed significant inter-clonal variability in canker lengths after 70 days but the natural prevalence of canker in each clone was not correlated with the inoculation results (French and Hart, 1978). This result is further evidence of the presence and differences in pre- and post-infection resistance mechanisms (Fig. 1).

The prevalence of stem cankers among trees in five clones in the field prior to the study had no correlation with canker expansion on inoculated trees of three of the clones (Enebak et al., 1999). However, greenhouse inoculations of grafted plants of the same five clones resulted in the same resistance ranking of the clones as the field inoculations. The authors concluded that selection of aspen clones for canker resistance in the field based on natural canker prevalence must be coupled with either artificial field or greenhouse inoculations to increase the likelihood of identifying canker resistant breeding parents.

Valentine et al. (1976) inoculated branches of aspen in 6 half-sib groups of 4 families each to study three potential mechanisms of resistance and their heritability. They found very low heritability among the groups for callus formation, branch death, and retardation of canker growth and each was influenced by the virulence of the pathogen genotype used. Examination of these mechanisms among trees in nine clones (Griffin et al., 1984) revealed a complex interaction of host-pathogen genetics and a high degree of independence of the three mechanisms among the aspen clones and fungal isolates tested.

The conflicting results of the above reports may have been caused by the experimental procedures used and confounded in part by differences in host vigor and inoculum potential (Bier, 1964). Uniformity in pathogenicity studies accounting for seasonal differences in moisture, nutritional and microbiological effects of bark on infection may reduce these problems in future tests.

After 20 years in a Minnesota planting of 586 aspen trees from 38 controlled pollinations no differences were detected in the incidence of Hypoxylon canker among progeny trees when both parents were diseased, only one parent diseased, or both parents disease-free (Anderson and Ostry unpublished). Diseased and disease-free parent trees propagated from root cuttings also had a similar incidence of Hypoxylon canker. This indicates that resistance to infection was not effective in the group of over 40 parent trees used in these crosses. After 40 years fewer than 10 of the 586 trees were canker-free and exhibit superior growth and form. Some of these trees have large numbers of oviposition wounds by *S. inornata* but all are callused-over. These trees may have resistance to canker owing to their response to wounding by insects, a pre-infection resistance mechanism, and may have value as breeding parents.

4. Conclusions

Many aspects of the Hypoxylon disease cycle have been clarified in the last 85 years including the mechanism of pathogenesis, the association of insects with infection, and several mechanisms of host resistance. Research groups using many methodologies have studied portions of this disease system in laboratories, growth chambers, greenhouses and in the field in plantations and natural stands of various ages across a wide range of sites. Each approach has added to our knowledge; however, the results were often contradictory or did not correlate well. Several major gaps in our understanding of the disease remain, specifically the influence of site and environment on disease incidence and severity. However, knowledge we have gained on the infection process has improved our ability for developing strategies for aspen improvement and management in native stands to minimize the impact of Hypoxylon canker.

Ascospores are the result of sexual recombination and are the primary source of inoculum of this disease. A second spore, previously thought to be infective asexual conidia, are now thought to function as spermatia (gametes) but this has not been proven experimentally. Two fungal traits, however, strongly supports this function: (1) the fungus produces receptive hyphae that receive the nucleus from the spermatia prior to hybridization and ascospore formation (Rogers and Berbee, 1964) and (2) an out breeding system and also a very strong somatic incompatibility system, prevents hyphal anastomosis between genetically different field isolates (Griffin et al., 1992). These genetically unique field isolates result from ascospore infections and since there are no asexual spores pathogenic races of the fungus cannot develop.

The cereal crops and the smut fungi are examples of diseases no longer a problem because of the effective breeding strategy for resistance. In these crop pathosystems, similar to the *E. mammata-Populus* system, the pathogens lack conidia, pathogenic races do not develop, the infective inoculum is a product of meiosis and functional resistance mechanisms are pre-infection. Plant breeders and pathologists have been quite successful in developing smut-free cereals and their methods and concepts may help in breeding for resistance to Hypoxylon canker. Up to the present time, most of the resistance mechanisms identified in this canker disease can be classified as post-infection (Fig. 1). These include callus formation, canker elongation rate, branch death, branch diameter, etc. In most cases these post-infection resistance traits do not correlate well with the natural prevalence of canker in a clone. The traits that may correlate to disease resistance are pre-infection and may include levels of defense compounds, callus formation, branch self-pruning, and resistance to various insects that provide entry courts for the fungus, all of which are traits that are difficult to select for.

Hypoxylon canker incidence and damage in the Lake States is the greatest in the first 20 years of a developing stand. This is the period when wood-boring insects can create wounds on low branches and when cankers on branches can expand into the lower main stem resulting in lethal cankers. This is in contrast to cankers originating in the upper crowns of older trees that result in non-lethal top cankers. Managers may want to monitor young stands for outbreaks of defoliating and wood-boring insects that may increase the vulnerability of stands to Hypoxylon canker.

The effect of thinning aspen on the incidence of Hypoxylon canker has been complicated by site and clone interactions. Long-term studies have shown that aspen genetics probably plays an important role in canker susceptibility. However, a strong case can be made that spatial resistance achieved by maintaining high stocking in young stands reduces stand vulnerability through early self-pruning and reduced populations of branch-wounding insects.

Possibly the least understood element of Hypoxylon canker centers around the extent of influence that site and environmental factors have on the incidence and severity of the disease. A potential complicating factor in studying site factors relative to Hypoxylon canker is that aspen ramets can share a common root

system for many decades (Tew et al., 1969) and individual ramets may be growing on microsites that are vastly different from each other. More importantly, any conclusions concerning site factors influencing canker incidence and severity must take into consideration clonal variation in canker susceptibility. Long-term common garden experiments with common clones grown across a range of sites and environments would greatly assist in explaining how various edaphic factors mediate the disease cycle.

Greenhouse gases, climate change and increased climate variability have the potential to influence the future incidence, severity and distribution of Hypoxylon canker as well as the distribution of aspen populations and populations of the various insects associated with this pathosystem. Longevity of aspen is known to be greater in the northern portions of its range and cooler air temperatures are associated with increased longevity potential in the Great Lakes Region (Shields and Bockheim, 1981).

Productivity of aspen was affected by increased levels of CO_2 and O_3 and the resulting changes in physical and chemical leaf defenses also affected insect and pathogen populations (Percy et al., 2002). How a changing environment and the many complex interacting biotic and abiotic factors will affect the resistance of aspen to *E. mammata* is unknown but increased generations of poplar borers caused by a warming climate could result in increased disease incidence and increased drought stress could enhance canker development (Bier and Rowat, 1962a; Griffin et al., 1986).

Climate change involving increased temperature should increase the incidence of the disease throughout the Lake States region. Both the pathogen and the insects that make wounds colonized by the fungus are favored by increased temperature. Most strains of the pathogen have optimum growth rates of 25–28 C which exceed current ambient temperatures in most areas. The incidence of the disease in the region is correlated with temperature (Anderson, 1952). In the northern counties of Minnesota the disease incidence on live trees is 1.8% and increases southward in the state. In the rest of the region the disease incidence ranges from 4.8 to 6.1% with the highest disease incidence in areas near the Great Lakes where weather is moderated. The life cycles and survival of insects known to provide entry courts for the fungus will be affected by increased temperatures. The life cycle of *S. inornata* ranges from 1 year in the southern areas to 2 years in more northerly areas (Nord et al., 1972a). Extreme cold temperatures during the winters of 1983 and 1985 resulted in dramatic decreases in the populations of *S. inornata* coincident with a reduction of new Hypoxylon cankers during the following several years within an aspen plantation (Ostry and Anderson unpublished).

Progress in a plant improvement program requires genetic knowledge of both the host and pathogen. Aspen selection and improvement for increased canker resistance has not been as successful as other tree species improvement efforts due in large part to the difficulty in accounting for non-specific wound responses of aspen clones. Pre- and post-infection factors have not been adequately accounted for and are probably responsible for much of the poor correlation of the results of artificial screening to the natural canker incidence among clones. In many cases inter-species crosses have not been successful with aspen because of their susceptibility to other damaging diseases.

Short- and long-term field studies of canker resistance of clones have not yielded consistent results caused by differences in the fate of infected trees. Short-term inoculation studies comparing differences in canker lengths do not reflect the potential for trees to recover or retard canker expansion over time. The prevalence of the disease in stands at any given time does not reflect the true incidence of the disease since many trees may have died earlier in the stand history so in the absence of reliable selection techniques, long-term monitoring is needed to properly evaluate clones and parents in an aspen improvement program.

Understanding the basis for resistance to *E. mammata* in natural stands will greatly enhance strategies for aspen management and genetic improvement. We believe identifying pre-infection factors for resistance in this pathosystem is critical. Molecular studies of pre-infection wound-inducible defense-related genes may provide a rapid way to screen for canker resistant clones.

Early research on host resistance centered on post-infection mechanisms. With our current understanding of the infection process, future aspen breeding lines should possess superior growth characteristics plus several sources of pre-infection resistance. These include rapid callus production to close insect oviposition wounds and, if possible, biochemical resistance to insect oviposition wounds. Today, based on several studies reviewed in this paper, aspen is protected from insect wounds and subsequent infection by *E. mammata* almost entirely by spatial resistance.

Aspen improvement for Hypoxylon canker resistance may not require that resistance genes remain functional for many decades. An improved aspen line or clone at a plantation spacing will be most susceptible to the greatest damage by the disease during the first 20 years. Dense sucker regeneration after the first harvest will provide the spatial resistance phenomenon which reduces populations of sun-loving wood boring insects and subsequently can reduce lower stem infection due to fewer oviposition wounds. We are optimistic about the possibilities for aspen improvement and management to reduce the impacts of this disease by applying the knowledge that we have gained over the last 85 years of the many interacting biological and genetical factors of this disease system.

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